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The harmful alga *Aureococcus anophagefferens* utilizes 19′-butanoyloxyfucoxanthin as well as xanthophyll cycle carotenoids in acclimating to higher light intensities

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ABSTRACT

Aureococcus anophagefferens is a picoplanktonic microalga that is very well adapted to growth at low nutrient and low light levels, causing devastating blooms ("brown tides") in estuarine waters. To study the factors involved in long-term acclimation to different light intensities, cells were acclimated for a number of generations to growth under low light (20 μmol photons m^{-2} s^{-1}), medium light (60 or 90 μmol photons m^{-2} s^{-1}) and high light (200 μmol photons m^{-2} s^{-1}), and were analyzed for their contents of xanthophyll cycle carotenoids (the D pool), fucoxanthin and its derivatives (the F pool), Chls c_2 and c_3, and fucoxanthin Chl *a*/*c* polypeptides (FCPs). Higher growth light intensities resulted in increased steady state levels of both diadinoxanthin and diatoxanthin. However, it also resulted in the conversion of a significant fraction of fucoxanthin to 19′-butanoyloxyfucoxanthin without a change in the total F pool. The increase in 19′-butanoyloxyfucoxanthin was paralleled by a decrease in the effective antenna size, determined from the slope of the change in F_0 as a function of increasing light intensity. Transfer of acclimated cultures to a higher light intensity showed that the conversion of fucoxanthin to its derivative was a relatively slow process (time-frame of hours). We suggest the replacement of fucoxanthin with the bulkier 19′-butanoyloxyfucoxanthin results in a decrease in the light-harvesting efficiency of the FCP antenna and is part of the long-term acclimative response to growth at higher light intensities.

1. Introduction

Aureococcus anophagefferens is a picoplanktonic member of the Pelagophyceae (Heterokonta) that is notorious for causing devastating blooms ("brown tides") in estuarine waters in the north-eastern USA, in some regions occurring annually [1–3]. In an *Aureococcus* bloom, cell densities can be as high as 10^6/ml in shallow waters, resulting in such severe shading as to cause the death of sea-grasses growing underneath. Its disturbance of the ecosystem and toxicity to some species have effectively wiped out the shell-fish industry in several areas. It thrives in waters with high turbidity and organic (reduced) nitrogen levels [1,3,4], and can reach maximum growth rates at lower light intensities than competing species of diatoms and prasinophytes from the same environment [3,4].

The draft genome sequence of *A. anophagefferens* (henceforth referred to as *Aureococcus*) showed that this alga is well-equipped for sustained growth in its estuarine environment [3]. It has many genes for assimilation of both inorganic and organic forms of nitrogen and phosphorus, as well as an impressive array of genes for transporting and degrading a great variety of organic compounds [3,5,6]. All of these should enable heterotrophic (or more properly, mixotrophic) growth under low light levels as well as survival in darkness, e.g. in sediments [7,8]. It also has more than 60 different genes for fucoxanthin Chl *a*/*c* proteins (FCPs) [3], members of the light-harvesting complex (LHC) superfamily [9], consistent with its ability to grow to high densities under low light levels. These include four members of the Lhcx clade (also called LI818 or LhcSR) known to be involved in photoprotection and stress response in other algae [10–16]. However, very little is known about the organization of the *Aureococcus* photosynthetic apparatus [17].

Although *Aureococcus* is considered to be genetically low-light adapted, it is known that cultures can acclimate to high light intensity if given enough time [4]. The ability to adjust to changes in light intensity is particularly important during the establishment of a bloom. We therefore asked what changes in pigments and pigment-binding proteins would be involved in the process of acclimation to changes in light intensity, and at what rate these changes would

Abbreviations: Chl, chlorophyll; Dd, diadinoxanthin; Dt, diatoxanthin; Fx, fucoxanthin; 19′-BFx, 19′-butanoyloxyfucoxanthin; LHC, light-harvesting complex; FCP, fucoxanthin Chl *a*/*c* protein; PAR, photosynthetically active radiation.

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occur. As well as the expected changes in xanthophyll cycle carotenoids [18–20] we found that acclimation to higher light intensities involved the conversion of a substantial fraction of the light-harvesting carotenoid fucoxanthin (Fx) to its derivative 19′-butanoyloxyfucoxanthin (19′-BFx). The latter pigment is considered an environmental marker for the presence of pelagophytes [2,21], although it is also found in some haptophyte species along with their characteristic derivative 19′-hexanoyloxyfucoxanthin [22,23]. In *Aureococcus*, we found that an increase in 19′-Bfx is correlated with a decrease in the calculated effective antenna size for PSII, and suggest that it plays an important role in the long-term light acclimation of these algae to higher light intensities.

2. Material and methods

2.1. Culture conditions

An axenic culture of *A. anophagefferens* (CCMP 1984) obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton was grown in ESAW medium [24] at 18 °C with gentle shaking at a light intensity of 60 μmol photons m^{-2} s^{-1} on a 12L:12D light:cycle (Philips F32T8TL841 daylight). Light intensities were measured with a Li-Cor model LI-189 with a SA quantum sensor. For acclimation to different light intensities, exponentially growing cells were inoculated into fresh medium and placed under different illumination conditions: low light (LL, 20 μmol photons m^{-2} s^{-1}), medium light (ML, 60 or 90 μmol photons m^{-2} s^{-1}) or high light (HL, 200 μmol photons m^{-2} s^{-1}). Light intensities were adjusted with neutral density screening, except for the HL cultures which were maintained in a separate chamber illuminated with Sanyo FL40SS-W/37 tubes. Cultures were kept under the same illumination conditions for six weeks with weekly dilution into fresh media after cell numbers were determined using a hemacytometer.

To study the response of acclimated cells to different light intensities, cells in mid-exponential phase were diluted into 1 l of fresh medium, grown to mid-exponential phase (1×10^7 cells/ml) and the cultures then divided into three flasks. Flasks from each starting irradiance were placed under low light (LL, 20 μmol photons m^{-2} s^{-1}), medium light (ML, 90 μmol photons m^{-2} s^{-1}) or high light (HL, 200 μmol photons m^{-2} s^{-1}) and sampled at the initial time point (time 0) and after 1 and 6 h.

2.2. Pigment analysis

Triplicate aliquots of 30 ml of algal cultures were harvested by filtration onto 25 mm GF/F filters, immediately frozen in liquid nitrogen, and stored at −80 °C. Two sets of independent experiments were tested. Pigments were extracted from the filters with 90% acetone and the extracts filtered through a 0.2 μm PTFE filter to remove cell debris. Sample volumes of 200 μl were injected automatically into the HPLC system. Pigments were separated on a C8 reverse-phase column as stationary phase (Uptisphere, 150×4.6 mm, 3 μm particle size, Waters). For the mobile phase, eluent A was a mixture of methanol:acetonitrile: aqueous pyridine (50:25:25) and eluent B was methanol:acetonitrile: acetone (20:60:20) as described in Zapata et al. [25]. The samples were analyzed using a Waters Alliance HPLC which included a 2796 separation module and a Waters Diode-array detector (350 to 750 nm). The HPLC was calibrated with 19 pure pigment standards purchased from DHI (Water and Environment, Horsholm, Denmark), which included chlorophyll *a*, chlorophyll c_2, chlorophyll c_3, fucoxanthin, 19′-butanoyloxyfucoxanthin, diadinoxanthin, diatoxanthin and ββ-carotene. Identification and quantification of the extracted pigments were done by comparison of the retention time, absorption spectra and peak area obtained with the standards.

2.3. Immunoblotting analysis

1 l of cells from each illumination growth condition (LL, ML and HL) was grown to mid-exponential phase (1.to 1.5×10^7 cells/ml), harvested by centrifugation at 6000 ×*g* and washed once with buffer A (50 mM Hepes, 10 mM KCl, 5 mM EDTA, 10% Glycerol, pH 8, supplemented with complete protease inhibitor cocktail (Roche)). The cell pellets were resuspended in 1 ml of buffer A and transferred to a 2 ml tube filled half-way with 0.3 mm glass beads. Cells were broken in a mini-bead-beater (Omni International) in 3 breakage cycles of 20 s each followed by a cooling step on ice. After a low speed centrifugation step to remove the glass beads, thylakoids were recovered by centrifugation at 14,000 ×*g* and resuspended in 0.5 ml of buffer A. 25 μl sample aliquots were used for total chlorophyll determination. The pigments were extracted with 90% acetone and their concentrations were determined by spectrophotometry (UV-1601PC, Shimadzu, Japan) according to the equations of Jeffrey and Humprey [26]. For immunoblotting, proteins were separated by SDS-PAGE on a 12–18% gradient gels and electrotransferred onto PVDF membranes (Millipore). 0.8 μg of total chlorophyll was loaded per gel lane. To detect the FCPs, two different antibodies were used: α-HaFCP, raised to the 19 kDa FCP of *Heterosigma akashiwo* and α-Lhcr, raised to the Chl *a* light-harvesting complex of the red alga *Porphyridium cruentum* (Agrisera). Detection was performed with ECL western blotting detection system (GE Healthcare).

2.4. Measurement of chlorophyll fluorescence and evaluation of related parameters

Chlorophyll fluorescence was measured with the portable fluorometer AquaPen (Photon Systems Instruments, Brno, Czech Republic, http://www.psi.cz), using the built-in protocols. Maximal quantum yield of photosystem II (PSII) photochemistry was determined as F_V/F_M, where $F_V=(F_M-F_0)$ is variable fluorescence. Nonphotochemical quenching was evaluated using the Stern–Volmer approach as $NPQ=(F_M-F_M')/F_M'$. The intensity of actinic light was 800 μmol photons m^{-2} s^{-1} and its duration was 200 s, which was sufficient to reach a steady-state Fm′. All values in Table 4 are the average of 3 separate samples.

The fast chlorophyll fluorescence rise (O–J–I–P transient) was measured using different intensities of 620 nm excitation light, detected using a 667–750 nm bandpass filter. The maximal intensity of excitation light provided by the fluorometer is 3000 μmol photons m^{-2} s^{-1} and the time of illumination at each light intensity was 2 s, with dark-adaptation of the sample for 4 min between each light intensity. This time was sufficient to open all the reaction centers. F_0 was determined using the fluorometer's built-in routine. F_0 is considered to be a result of the so called transfer equilibrium [27], which is defined as the equilibrium between the formation of excited states among all light-harvesting pigments and utilization of the excited states for reversible primary photochemistry. A larger antenna size should therefore mean a larger number of excitons in equilibrium. The effective antenna size was therefore defined as the slope of the change in F_0 as a function of increasing light intensity [28,29].

3. Results

3.1. Acclimation of cell cultures to different light intensities

Cultures were acclimated for 6 weeks to growth under four different light intensities: low light (20 μmol photons m^{-2} s^{-1}), medium light (60 or 90 μmol photons m^{-2} s^{-1}) and high light (200 μmol photons m^{-2} s^{-1}) with weekly transfers to fresh medium in exponential phase before gathering the data in Fig. 1. Cells grown under medium light are in the range where photosynthesis is saturated but cells should not be experiencing appreciable amount of high light stress [4]. Cultures "acclimated" to 200 μmol photons m^{-2} s^{-1}

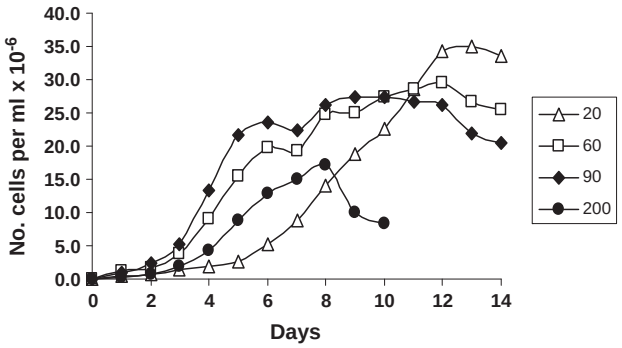


Fig. 1. Growth of *Aureococcus* cultures at four different light intensities. Cells acclimated for six weeks to low light (LL, 20 μmol photons m^{-2} s^{-1}), medium light (ML, 60 or 90 μmol photons m^{-2} s^{-1}) and high light (HL, 200 μmol photons m^{-2} s^{-1}) were diluted into fresh media at day zero and cell counts determined every day. Key: light intensity at μmol photons m^{-2} s^{-1}.

(HL) stopped growing at a lower cell density and remained pale yellow, in contrast to the yellowish-brown and greenish-yellow colors of the low light (LL) and medium light (ML) cultures.

3.2. Photosynthetic pigments in acclimated cell cultures

Total pigments were extracted from cells in mid-exponential phase, at a cell density of approximately 1.5×10^7 cells/ml. Pigments were separated by reverse-phase-HPLC using a C8 column and identified by comparison of their spectra and retention times to pure standards. The prominent pigments were those characteristic of pelagophytes [2,25]: Chls *a*, c_2, c_3, β-carotene, diadinoxanthin (Dd), diatoxanthin (Dt), fucoxanthin (Fx) and 19′-butanoyloxyfucoxanthin (19′-BFx) (Fig. 2). All the identified pigments were found under all three conditions, but with significant differences in ratios, particularly Dt/Dd and 19′-BFx/Fx. Note that the scales of the y-axes are different, because the amounts of pigment per cell are different (Table 1). Trace amounts of violaxanthin, antheraxanthin and zeaxanthin were detected only in cells acclimated to HL. A pigment eluting just after diadinoxanthin (b) was tentatively identified as a fucoxanthin derivative according to its spectrum, but it does not correspond to any commercially available standard.

Quantification of pigments normalized to Chl *a* (Fig. 3) showed that there were only low levels of Dt in LL and ML cultures, suggesting that the algae were truly acclimated and not experiencing light stress. In fact, in some experiments the level of Dt under LL conditions was too low to be quantified. The Dd level (Fig. 2, peak 5) was much higher in ML than LL cells, and was even higher in HL cells. There was very little Dt (Fig. 2, peak 6) in ML cells but a substantial amount in cells grown under HL. This was not due just to conversion of Dd to Dt, because both were increased in comparison to ML cells. Overall, the total D pool (Dd plus Dt) increased by a factor of 3 from LL to ML, and doubled again under HL growth (Table 2). There was very little difference between the two ML conditions (60 and 90 μmol photons m^{-2} s^{-1}).

As expected, the pigments primarily involved in light-harvesting such as Fx and the Chls *c* were highest under LL and lower under higher light. However, the decrease of Fx under ML was paralleled by an increase in its derivative, 19′-butanoyloxyfucoxanthin (19′-BFx). The increase in 19′-BFx was almost equal to the decrease in Fx, with the exception of cells cultivated under HL. There was very little difference between the treatments in the sum of Fx and 19′-BFx ("F Pool") (Table 2), suggesting that Fx was being converted to 19′-BFx rather than the latter being synthesized de novo. Chlorophyll c_3 was highest under LL, decreased more than 50% under ML and decreased even more under HL, whereas Chl c_2 was not as strongly affected. Under

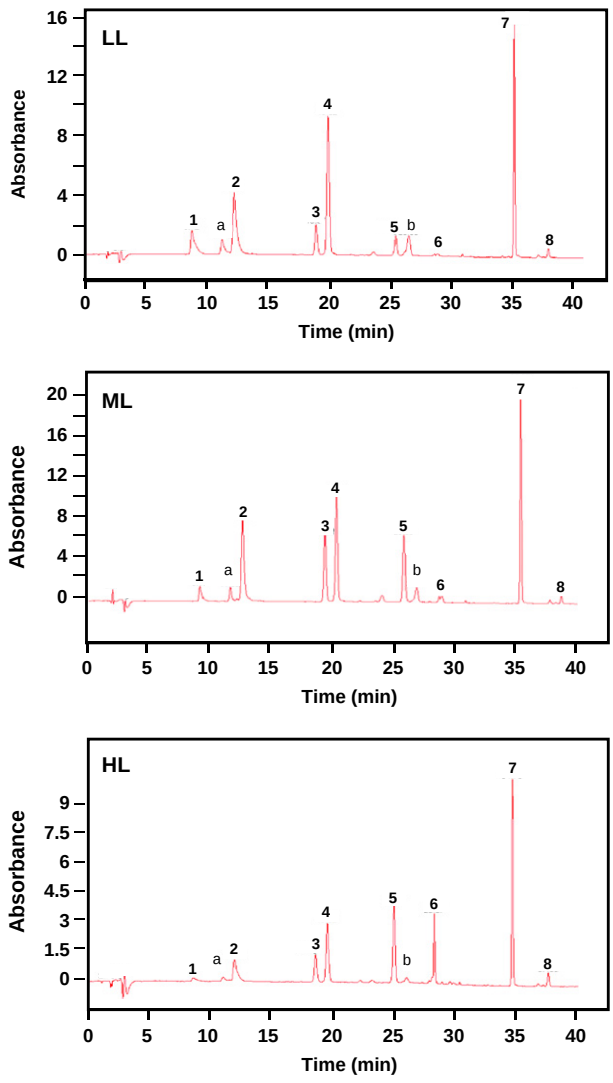


Fig. 2. HPLC traces of pigment separation from *Aureococcus* cells grown under LL, ML (60 μmol photons m^{-2} s^{-1}) or HL. No significant differences were observed between the elution profiles of cells grown under 60 and 90 μmol photons m^{-2} s^{-1} (data not shown). Peak numbers: 1, Chl c_3; 2, Chl c_2; 3, 19′-butanoyloxyfucoxanthin, 19′-BFx; 4, fucoxanthin, Fx; 5, diadinoxanthin, Dd; 6, diatoxanthin, Dt; 7, Chl *a*; 8, β-carotene, β-car. Minor peaks a and b are chlorophyllide *a* and an unknown carotenoid pigment respectively. Note differences in scale.

HL, both fucoxanthins as well as both Chls *c* decreased relative to Chl *a*, representing a general down-regulation of light-harvesting under HL as found in other systems [e.g. 19,20].

Table 1
Photosynthetic pigments (on the basis of cell numbers) of cultures acclimated to growth under different light intensities. Values are the mean (s.d.) of triplicate samples expressed as 10^{-18} mol/cell.

	Light intensity (μmol photons m^{-2} s^{-1})			
Pigment	LL (20)	ML (60)	ML (90)	HL (200)
Chl *a*	20.28 ± 1.91	18.24 ± 1.55	19.59 ± 0.98	9.43 ± 0.19
Chl c_3	3.75 ± 0.30	1.38 ± 0.06	1.73 ± 0.08	0.28 ± 0.02
Chl c_2	7.94 ± 0.50	6.83 ± 0.24	6.86 ± 0.26	1.67 ± 0.16
Fx	15.54 ± 0.18	9.79 ± 0.90	10.97 ± 0.11	3.76 ± 0.12
19′-BFx	3.53 ± 0.43	5.90 ± 0.47	5.59 ± 0.12	1.69 ± 0.05
Dd	1.91 ± 0.14	5.79 ± 0.49	5.93 ± 0.13	4.21 ± 0.20
Dt	0.12 ± 0.01	0.30 ± 0.06	0.37 ± 0.03	2.26 ± 0.10
β-car	0.29 ± 0.02	0.25 ± 0.02	0.26 ± 0.01	0.23 ± 0.01

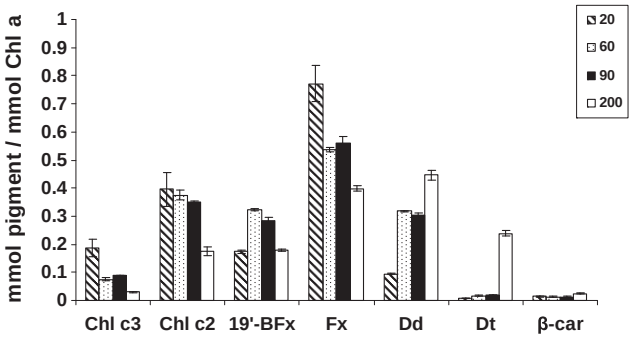


Fig. 3. Photosynthetic pigments of cells acclimated to growth at low, medium and high light intensities, normalized to Chl *a*. Hatched, 20 μmol photons $m^{-2} s^{-1}$; dotted, 60 μmol photons $m^{-2} s^{-1}$; black, 90 μmol photons $m^{-2} s^{-1}$, white, 200 μmol photons $m^{-2} s^{-1}$.

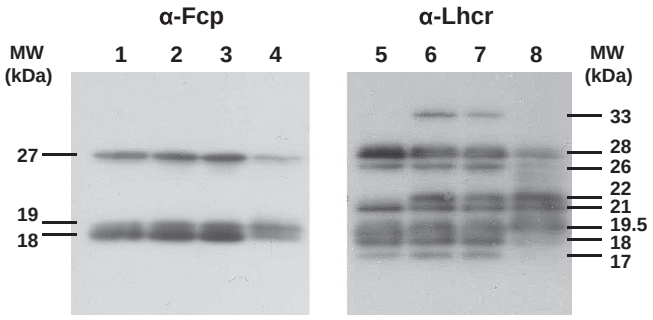


Fig. 4. Separation of thylakoid proteins on a 12–18% SDS-PAGE from cells acclimated to LL (lanes 1 and 5: 20 μmol photons $m^{-2} s^{-1}$), ML (lanes 2 and 6: 60 μmol photons $m^{-2} s^{-1}$ and lanes 3 and 7: 90 μmol photons $m^{-2} s^{-1}$) or HL (lanes 4 and 8: 200 μmol photons $m^{-2} s^{1}$) and visualized by immunoblotting with α-FCP (lanes 1–4) or α-Lhcr (lanes 5–8).

3.3. LHC polypeptides

To determine whether these large differences in pigment composition were correlated with changes in LHC polypeptides, thylakoids isolated from cells acclimated to these four light intensities were solubilized and the polypeptides separated by SDS-PAGE followed by immunostaining with two antisera known to demonstrate cross-reactivity with other members of the LHC family due to sequence relatedness [9]. The antibody raised to one of the major FCP polypeptides of *H. akashiwo* immunostained three prominent bands of 18, 19 and 27 kDa (Fig. 4). Based on their calculated molecular weights, the two broad bands at about 18–19 kDa are the major Lhcf polypeptides that are commonly reported in other heterokont algae [16].

The α-Lhcr antibody, raised to a red algal PSI-associated LHC protein, decorated at least eight immuno-reacting polypeptide bands. This does not mean that only eight of the many LHC genes in *Aureococcus* are expressed, because many of them encode polypeptides that are closely related in sequence and may have the same apparent molecular weight in the mature form. The immunoblot showed that polypeptides of 22 and 33 kDa are present in cells acclimated to ML (lanes 6 and 7) but not those acclimated to LL (lane 5). Under HL growth conditions, the 22 kDa band persisted but the relative intensities of most other bands were decreased and the 17, 18 and 33 kDa bands disappeared entirely. Note that the gel lanes were loaded on an equal Chl basis because the amounts of Chl/cell and Chl/protein were markedly diminished under HL (Table 1 and data not shown). There were no bands that were noticeably higher under HL and would be candidates for Dt-binding [16].

3.4. The early stages of acclimation to different light intensities

In order to understand how *Aureococcus* acclimates to higher light intensity at the molecular level, cultures well-acclimated to LL and ML (90 μmol photons $m^{-2} s^{-1}$ only) were transferred to ML or HL (200 μmol photons $m^{-2} s^{-1}$) at the beginning of the light period (time 0 h) and sampled for pigment analysis after 1 and 6 h. To control for diel effects [30], cultures left at their original light intensity were sampled at the same time points. In LL acclimated cultures, there was a small increase in the Dd + Dt pool ("D pool") during the day, due entirely to Dd (Fig. 5B). In ML acclimated cultures, there was an increase in Dd after 1 h illumination, along with a small transient increase in Dt that had almost disappeared by the 6 h time point (Fig. 5C, note difference in scale).

After transfer to higher light, there were increases in both Dd and Dt in the first hour. In LL acclimated cultures moved to ML, both D pool carotenoids were further increased after 6 h. After transfer to HL, the D pool did not increase after the first hour, but there was a large conversion of Dd to Dt, equivalent to 50 mmol Dt/mol Chl *a* (Fig. 5B,C). In contrast, ML acclimated cells started with a much larger D pool and a small but significant amount of Dt. Comparing cells left at ML with those exposed to HL for 6 h, there was no significant increase in Dd but a large increase in Dt. However, the increase in Dt was actually less significant than the changes for cells acclimated to LL and shifted to HL.

It appears that acclimation to lower light intensity is a slower process than acclimation to higher light intensities. When LL cells were up-shifted up to ML, the cells achieved the same level of D pool carotenoids as the ML acclimated cells within 6 h. However, when cells acclimated to ML were down-shifted to LL, the D pool only decreased by 20% in 6 h, and was still twice the level of cells acclimated to LL (Fig. 5A). Although the absolute amount of Dt decreased, the Dt level never reached the almost undetectable levels of cells acclimated to LL. We did not do a similar down-shift experiment with HL cells because their Chl content was so low (Table 1) and the amount of Dt so high that we were not convinced that they were truly acclimated [4].

There was almost no difference in total fucoxanthin carotenoids (F pool) between cells acclimated to LL or ML, and little change after their transfer to higher light (Table 3). However, there was a three-fold difference between LL and ML acclimated cells in the fraction of the F pool due to 19′-BFx (Fig. 5D, Table 3). Transfer of LL cells to ML or HL for 6 h caused an increase of at least 50% in the fraction of the F pool due to 19′-BFx. Similarly, when ML cells were transferred to HL, the fraction of 19′-BFx increased by 35%, without a corresponding increase in pool size, although some of this increase was apparently a diel effect (see cells left at ML). These data suggest that Fx is being converted into 19′-BFx rather than being synthesized de novo, in contrast to the xanthophyll cycle carotenoids. However, the rate of this conversion appears to be relatively slow, since even after LL cells spent 6 h at ML, the amount of 19′-BFx was only 192 mmol/mol Chl *a*, which is far lower than the value of 320 mmol/mol Chl *a* for cells acclimated for 6 weeks to ML. There was no significant change when ML cells were transferred down to

Table 2
Pigments of acclimated cultures normalized to Chl *a* (mmol $mmol^{-1}$), showing pool sizes. Calculated from data in Table 1.

	Light intensity (μmol photons $m^{-2} s^{-1}$)			
Pigment	LL (20)	ML (60)	ML (90)	HL (200)
Fx	0.771 ± 0.064	0.537 ± 0.009	0.561 ± 0.024	0.398 ± 0.009
19′-BFx	0.173 ± 0.005	0.323 ± 0.004	0.286 ± 0.009	0.179 ± 0.004
F pool	0.884	0.860	0.847	0.577
Dd	0.094 ± 0.002	0.317 ± 0.001	0.303 ± 0.009	0.446 ± 0.018
Dt	0.006 ± 0.001	0.017 ± 0.002	0.019 ± 0.001	0.239 ± 0.011
D pool	0.100	0.334	0.322	0.685
Chl c_2	0.395 ± 0.059	0.375 ± 0.018	0.351 ± 0.004	0.177 ± 0.016
Chl c_3	0.187 ± 0.031	0.075 ± 0.005	0.088 ± 0.002	0.030 ± 0.001
Total Chl *c*	0.582	0.450	0.439	0.207
β-car	0.014 ± 0.001	0.013 ± 0.001	0.013 ± 0.001	0.024 ± 0.001

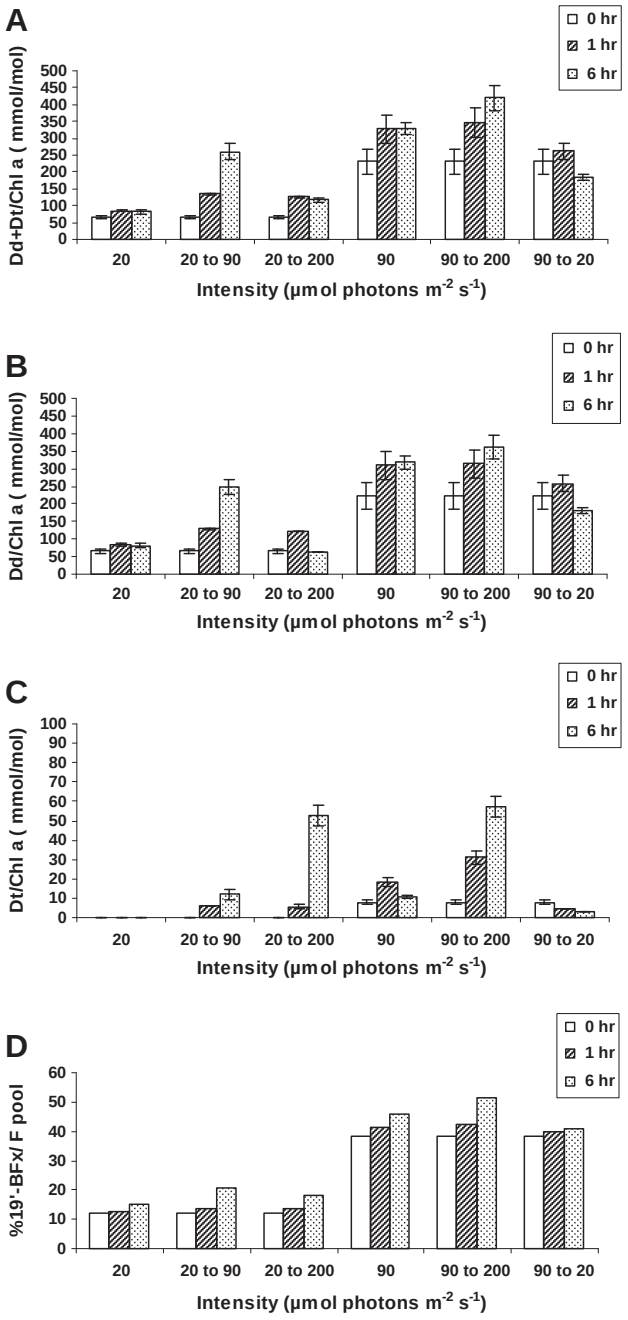


Fig. 5. Pigment response to a change in light intensity. At time zero (white bars), cells acclimated to 20 or 90 µmol photons m^{-2} s^{-1} were transferred to the indicated light intensities and sampled after 1 h (hatched bars) or 6 h (dotted bars). (A) D pool (Dd + Dt), (B) Dd, and (C) Dt, normalized to Chl *a*. (D) 19′-BFx as % of F pool.

LL for 6 h. As with the xanthophyll carotenoids, it appears that acclimation to a decrease in light intensity takes longer than acclimation to an increased light intensity.

3.5. Effect of culture age on steady-state pigment levels

In the process of repeating these experiments, we discovered that although cultures in different growth phases had the same response to high light, the magnitude of the response depended on whether the cultures were in mid or late-exponential phase (data not shown). Fig. 6A shows the relative amounts of 19′-BFx and Fx in cells acclimated to 20, 60 and 90 µmol photons m^{-2} s^{-1} but sampled in mid-exponential phase (left panel, data from Fig. 3 and Table 1) or stationary phase (right panel, from an independent experiment). It shows that the

Table 3
Fucoxanthin and 19′-butanoyloxyfucoxanthin during early stages of acclimation to higher light (mean of triplicate samples).

	Pigments (mmol per mol Chl *a*)			
Light regime (µmol photons m^{-2} s^{-1})	Fx	19′-BFx	F pool (Fx + 19′-BFx)	% 19′-BFx
20 acclimated	757 ± *31*	106 ± *02*	863 ± *83*	12.3
20 to 90, 1 h	747 ± *23*	116 ± *05*	863 ± *27*	13.4
20 to 90, 6 h	726 ± *75*	192 ± *33*	918 ± *108*	20.9
20 to 200, 1 h	745 ± *08*	116 ± *02*	860 ± *10*	13.4
20 to 200, 6 h	720 ± *35*	158 ± *07*	878 ± *42*	17.9
90 acclimated	513 ± *44*	320 ± *37*	833 ± *84*	38.4
90 to 200, 1 h	534 ± *53*	390 ± *38*	924 ± *91*	42.2
90 to 200, 6 h	404 ± *28*	425 ± *34*	829 ± *62*	51.3
90 to 20, 1 h	554 ± *53*	370 ± 36	924 ± *89*	40.1
90 to 20, 6 h	511 ± *15*	350 ± *12*	861 ± *26*	40.6

amount of 19′-BFx is much higher in stationary phase than mid-exponential phase, as originally reported in the paper that defined the Pelagophyceae [2]. In our experiments, 75% of the Fx in ML cells is converted to 19′-BFx in stationary phase versus 30% in mid-exponential phase. However, the total pool of Fx is little decreased in stationary phase compared to mid-exponential phase.

The story is less striking when it concerns the D pool (Fig. 6B). Stationary phase cultures have increased amounts of both Dd and Dt compared to mid-exponential cultures. Cells well-acclimated to the three growth light conditions have negligible amounts of Dt (approximately 5–6% of the D pool) in mid-exponential phase regardless of

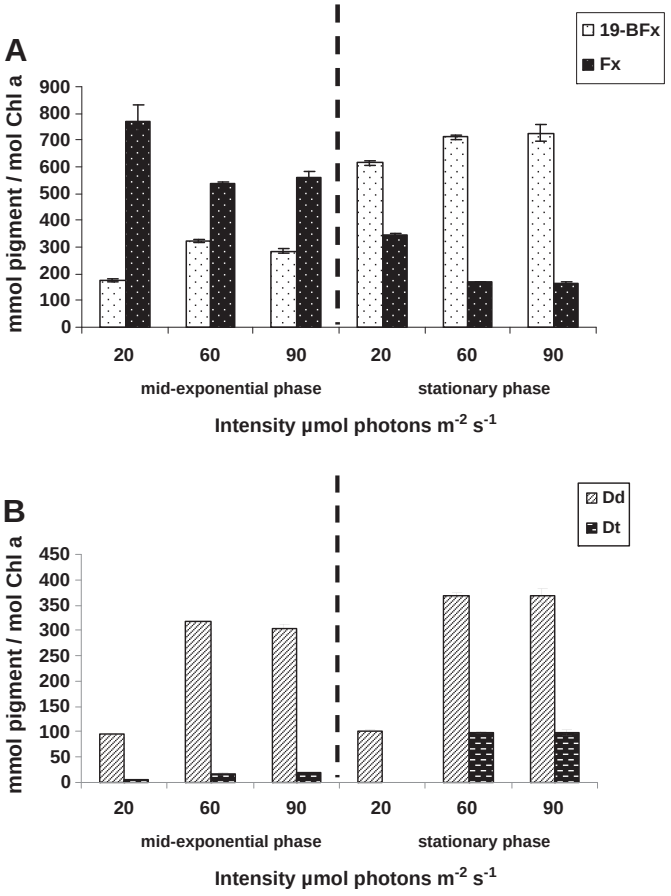


Fig. 6. Effect of growth phase on pigment ratios under LL and ML. A: Fx (black bars) and 19′-BFx (dotted bars). B: Dd (hatched bars) and Dt (black bars). All pigments were normalized to Chl *a*.

light intensity, whereas there is a much higher fraction of Dt (about 25%) in stationary phase cells.

3.6. Determination of effective antenna size

Non-photochemical quenching (NPQ) and the maximum quantum yield of photosystem II photochemistry (Fv/Fm) were determined for cultures acclimated to LL and ML, using the built-in routines of the portable AquaPen fluorometer (Table 4). Cells acclimated to the higher light intensity had a slightly lower Fv/Fm but were still in the range typical for healthy phytoplanktonic algae. They also showed a higher level of NPQ, consistent with their increased content of xanthophyll cycle carotenoids.

The effective antenna size was defined as the slope of the line relating F_0 to the energy of the excitation light [27,29]. F_0 reflects the equilibrium between the formation of excited states in the light-harvesting pigments and their utilization for photochemistry when all the reaction centers are open [27,28]. Since both photosystems contribute to F_0 [29], the calculated antenna size represents the total antenna size. Since stress conditions can cause closure of PSII reaction centers, this approach is only suitable for cells that are well-acclimated to their environmental conditions and not for stressed samples, i.e. cultures that have been transferred to a higher light intensity for only a few hours, such as those in Fig. 5.

The results for one experiment are shown in Table 4, along with the proportions of Fx and 19′-BFx in the cultures used for that experiment. The antenna size calculated for LL cells was 63 (arbitrary units) compared to 49 (arbitrary units) for ML cells, i.e. a ratio of slopes of 1.29. In a second experiment the values were different but the ratio was almost the same (1.34), so in both cases the antenna size calculated by this method was 30% higher in LL cells than in ML cells.

4. Discussion

These experiments show that *Aureococcus* employs strategies involving several carotenoids in acclimating to changes in light intensity, and that they operate on different time scales. Because we were interested in the processes of acclimation rather than the immediate stress response, we did not study the very fast initial conversion of Dd to Dt without net synthesis, which occurs in the first minutes of HL exposure and is believed to be responsible for the qE component of non-photochemical quenching [10,18,31]. Over the first few hours of higher light exposure, *Aureococcus* showed the next level of response: a steady increase of both Dd and Dt, characteristic of the beginning of acclimation to higher light intensity in diatoms and other marine algae [11,13,20]. On an even slower time scale, *Aureococcus* converts a fraction of the light-harvesting carotenoid Fx to its 19′-butanoyloxy derivative (Fig. 5). The full extent of this conversion was only seen in cultures completely acclimated to the higher light conditions. Upon transfer back to lower light intensities, the conversion of 19′-BFx to Fx occurred more slowly than the initial conversion of Fx to 19′-BFx under high light exposure. The same seems to be true for the conversion of Dt back to Dd.

Table 4
Photosynthetic parameters and F pool pigments.
(Pigment data from the experiment of Fig. 5 and Table 3).

	Acclimation light intensity (μmol photons m^{-2} s^{-1})	
	20	90
Fv/Fm (±s.d.)	0.674 (±0.005)	0.590 (±0.006)
NPQ (±s.d.)	0.377 (±0.016)	0.450 (±0.029)
% Fx in F pool	87.7	61.1
% 19BF in F pool	12.3	38.4
Effective antenna size (arbitrary units)	62.5	49.1

In terms of light-harvesting polypeptides, *Aureococcus* cells acclimated to ML had polypeptides of 22 and 33 kDa which were not present or were at extremely low levels in LL acclimated cells. In the absence of specific antibodies for the individual LHC proteins, we were unable to determine if they were members of the Lhcx clade, which is involved in photoprotective responses in diatoms [13,15]. However, the fact that the 33 kDa band disappeared in cells cultivated for 6 weeks under HL, while the 22 kDa band showed no further increase, suggested that neither polypeptide is specifically responsible for binding the high levels of D pool carotenoids in HL cells. We were unable to detect any consistent changes in band pattern when cells were shifted to a higher light for only 6 h, possibly due to the very large number (60+) of LHC family members in this alga [3]. Improved resolution of these polypeptides and their involvement in accessory pigment binding is a major goal of our ongoing research program.

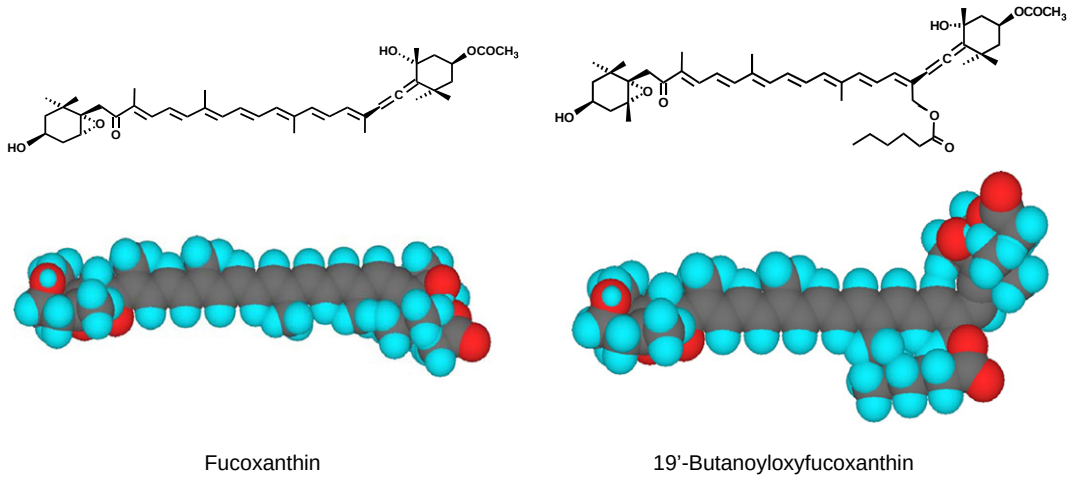
We suggest that *Aureococcus* follows a three-phase strategy in the process of acclimating to higher light conditions: the initial rapid conversion of Dd to Dt without net synthesis (minutes), an intermediate phase marked by net synthesis of both Dd and Dt and the conversion of Fx to19′-BFx (hours), followed by a slow phase that involves a continued increase in the fraction of 19′-BFx as well as changes in the light-harvesting protein composition and other physiological adjustments. According to McIntyre et al. [4], this last phase could take from days to weeks to finally reach the fully acclimated state, depending on the light intensity. The concept of three phases of high light acclimation has also been proposed in a comprehensive study of high light response in the diatom *Phaeodactylum tricornutum* (13).

Light intensity is not the only factor. There was also a marked increase in 19′-BFx and in the magnitude of its response to high light when cells were in stationary as opposed to exponential phase, as already noted in one of the first papers on *Aureococcus* [2]. As a general rule, cells proceed into stationary phase when the culture medium nutrients, primarily nitrogen, become limiting. Nutrient deprivation can result in high light stress due to lack of electron acceptors [18–20], which would explain the increased levels of 19′-BFx.

19′-BFx is considered to be an important environmental marker for the Pelagophyceae, the stramenopile group to which *Aureococus* belongs [2,21]. However, it is also found in trace amounts in some haptophyte species, although 19′-hexanoyloxyfucoxanthin is usually the predominant fucoxanthin derivative [22,32–34]. van Leeuwe and Stefels found that an Antarctic strain of the haptophyte *Phaeocystis* converted Fx to both 19′-BFx and the 19′-hexanoyloxy derivative under high light, and this conversion amounted to more than 50% of the F pool under iron limitation conditions [33]. These authors suggested that the acyloxy derivatives might affect the thylakoid membrane structure and result in less efficient light-harvesting, which would reduce the possibility of photodamage, particularly under Fe stress conditions [33].

In *Aureococcus*, we found a decrease in the calculated effective antenna size in ML cells with higher levels of 19′-BFx than LL cells (Table 4). Using light intensities very similar to those used in our experiments, Pustizzi et al. [30] showed that both the absorption cross section per cell and photosystem II efficiency were lower when *Aureococcus* was grown at 90 μmol photons m^{-2} s^{-1} compared to 25 μmol photons m^{-2} s^{-1}. Although our work was preliminary and subject to the limitations of the available instrumentation, both these pieces of evidence support the idea that the substitution of 19′-BF for Fx in the light-harvesting complexes may make the transfer of light energy to the reaction centers less efficient [33].

Space-filling models of the two carotenoids suggest a reason for this effect (Fig. 7). The addition of the butanoyloxy group at the 19′-position makes the molecule much more bulky, which could affect binding and conformation of the protein. Increasing distances between Chls and carotenoids and modification of the electrostatic



Fucoxanthin 19'-Butanoyloxyfucoxanthin

Fig. 7. Structures and space-filling models of fucoxanthin and 19′-butanoyloxyfucoxanthin. Space-filling models courtesy of Dr. Harry Frank.

environment around individual pigments might thus decrease the efficiency of energy transfer. In the diatom complexes that have been studied by resonance Raman spectroscopy, three different environments for fucoxanthin can be distinguished [35]. However, there is no information as to what residues are binding the fucoxanthin. Future studies will use spectroscopic techniques such as resonance Raman to gain insight into the effect of 19′-BFx on energy transfer within the light-harvesting antenna of *Aureococcus*.

Our data suggest that one of the reasons why *Aureococcus* is able to become so dominant under "bloom" conditions is that it has a more sophisticated acclimation strategy than the competing diatoms. It co-occurs with the diatom *Thalassiosira pseudonana* which has a smaller number of genes for LHC polypeptides (about 40), although the two algae do have the same number of the stress-related *Lhcx* genes [3,13]. The *Aureococcus* genome sequence now gives us the tools to investigate how the pigments and their polypeptides are involved in the acclimation of this alga to different light regimes, and to understand its success in the environment.

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